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(54) Title: PROCESS FOR OBTAINING PURE PEPTIDE TRANSFER FACTOR, TRANSFER FACTOR THUS OBTAINED AND USES THEREOF

(57) Abstract

The invention relates to a process for obtaining purified transfer factor, the transfer factor thus obtained, and uses thereof. The transfer factor is obtained via filtering a sample to remove proteins weighing less than 15 kilodaltons, followed by separation of the transfer factor via interaction with an antigen to which it is specific. Following separation of the complex, the transfer factor is subjected to reversed phase high performance liquid chromatography and then gel filtration high performance liquid chromatography. The resulting transfer factor is antigen specific, has a molecular weight of from about 4900 to about 5500 daltons, affects transfer of a delayed type cell mediated immune response to an antigen, but does not affect antibody mediated response.

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PROCESS FOR OBTAINING PURE PEPTIDE TRANSFER FACTOR, TRANSFER FACTOR THUS OBTAINED AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to transfer factor ("TF" hereafter). More particularly, it relates to processes for obtaining transfer factor, the transfer factor itself, and methods of using the material.

BACKGROUND AND PRIOR ART

Transfer factor ("TF") is defined by Stedman's Medical Dictionary as "a substance, free of nucleic acid and antibody, that is obtained from the leukocytes of a person with a delayed type sensitivity and that will, following injection into the skin of a nonsensitive person, transfer the specific sensitivity to the recipient". Illustrated Stedman's Medical Dictionary, 24th edition, page 511 (William & Wilkins, 1982). As amplified upon by Kirkpatrick et al., in Torrance, ed. Biological Response Modifiers pg. 329-359 (Academic Press, 1985), the disclosure of which is incorporated by reference, TF is a dialyzable substance obtained from leukocytes, usually lysed, of humans and other vertebrates that have been sensitized so as to express delayed type hypersensitivity or other cell mediated responses to a sensitizing antigen. TF binds homologous antigen and has the capacity to mediate transfer of delayed type hypersensitivity and other cell-mediated immune responses from one individual to another. In such a situation the individual from whom the TF is obtained has been sensitized to the antigen of interest. Notwithstanding the above properties, the TFs are smaller than antibodies, and do not transfer antibody mediated responses, nor do they induce antibody production. Kirkpatrick et al. supra; Kirkpatrick et al., J. Immunol. 134: 1723-1727 (1985); Peterson et al., J. Immunol. 126: 2480-2484 (1981); Burger

et al., Cell Immunol. 29: 410-413 (1977). These properties of transfer factor are also described by Spitler et al., U.S. Patent No. 3,991,182, which discusses a "transfer factor" secured from the leukocytes of healthy donors. The material suppresses disease symptoms. Spitler et al. describe the material as being heat stable, and having a molecular weight of less than 20,000. It is secured by lysing leukocytes, and then incubating the lysate with Mg^{2+} and DNase, followed by filtration through a millipore filter.

There have been numerous additional attempts to characterize the substance referred to as TF, these being reported in both the scientific and patent literature. Baram et al., J. Allergy 33: 498-506 (1962) fractionated human leukocyte extracts through ion exchange chromatography, using diethylaminoethyl cellulose (DEAE). This work was continued and, as reported by Baram et al., J. Immunol. 97: 407-420 (1966), gel filtration and paper chromatography were used. Among the conclusions presented by this work was the submission that TF contained nucleosides. Work by Lawrence et al., J. Clin. Invest. 34: 219-232 (1955); J. Exp. Med. 104: 321-323 (1956); Trans. Assoc. Amer. Physicians 76: 84-89 (1963), using gel filtration chromatography on leukocyte extracts of sensitized humans, led to a proposal that TF is (i) water soluble, (ii) dialyzable, (iii) has a molecular weight of less than 10,000, (iv) was resistant to deoxyribonuclease, ribonuclease and trypsin digestion, and (v) possessed a chromatogrpahic peak showing greater absorbance at 260 nm than at 280 nm. This combination of factors led to a proposal that TF was a small, ribonuclease resistant polyribonucleotide. The inclination toward the assumption that a nucleotide or nucleoside was a part of the TF molecule was continued by Gottlieb et al., Lancet 2: 822-823 (1973); and in U.S. Patent Nos. 4,468,379 and 4,616,079, Gottlieb differentiated transfer factors from immune modulators (the '379 patent), and amplifiers (the '079

patent). In the Lancet publication, Gottlieb postulated that TF consisted of 12 amino acids and an oligonucleotide. As a result, research focused on the study of eluates at wavelengths of 254 nm or greater. Many reports noted high 254/260 nm to 280 nm absorbance ratios, again suggesting oligonucleotides as part of the TF fraction. See, e.g., Arala-Chaves et al., Int. Arch. Allergy 31: 353-365 (1967); Neidhart et al., Cell Immunol. 9: 319-323 (1973); Reymond et al., Vox sang. 29: 338-351 (1975); Dunnick et al., Proc. Natl. Acad. Sci. USA 72: 4573-4576 (1975); Vandenbark et al., J. Immunol. 118: 636-641 (1977); Dunnick et al., J. Immunol. 118: 1944-1950 (1977); Burger et al., J. Immunol. 122: 1091-1098 (1979); Wilson Trans. Assoc. Amer. Physicians 92: 239-256 (1979); Borvak et al., Acta Virol. 29: 119-128 (1985). Similarly, Warren, U.S. Patent No. 4,435,384, hypothesizes a molecule of a molecular weight of from 5-10,000 containing protein and RNA. See also Goust et al., U.S. Patent No. 4,001,080, describing dialysable TF as a mixture of molecules of molecular weight generally from 4 to 7 kilodaltons and containing a ribonucleotide. Again, Wilson et al., U.S. Patent No. 4,816,563 describes three forms of transfer factor, all of which contain a nucleotide moiety and a peptide moiety. Note column 11 of this reference.

It will be seen that the prior art in this field has suggested that TF is a nucleotide/protein complex. Interest in the molecule and its structure has, if anything, increased because of its therapeutic efficacy. Apart from therapeutic uses described by the references set forth supra, reference may be made, e.g., to Viza et al., European Patent Application 101,200, suggesting TF therapy for HSV (herpes simplex virus). One also notes Warren, U.S. Patent No. 4,435,384, describing dermatological efficacy for blemishes, acne, condyloma and HSV. The material has been shown to be efficacious against C. albicans, as per Kirkpatrick et al. in Khan et al., ed., Immune Regulators In Transfer Factor, pg. 547-559 (Academic Press, 1979), the

disclosure of which is incorporated by reference. Additional showings of efficacy against Herpes simplex may be found at Khan et al., Dermatologica 163: 177-185 (1981); Dwyer in Kirkpatrick et al., Immunobiology of Transfer Factor, pg. 233-243 (Academic Press, 1983); Viza et al., Lymphokine Res 4: 27-30 (1985). Varicella zoster has been prevented with TF as per Steele et al., New Eng. J. Med. 303: 355-359 (1980). Louie et al., Clin. Immunol. Immunopath 44: 329-334 (1987) and McMeeking et al., J. Infect. Dis. 161: 108-112 (1990), showed efficacy against cryptosporidiosis in AIDs patients.

It has now been found, in contrast to the research described supra, that TF is not a peptide/nucleotide complex, but is a peptide. The invention described herein sets forth a process by which substantially pure peptide TF is obtained, the pure peptide itself, as well as methods for using the resulting molecule. The achievement of these inventions is amplified upon in the disclosed which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic of the strategy used to purify transfer factor.

Figure 2 shows dose-response relationships for dialysates of lysed splenocytes which contain TF.

Figure 3 shows the dose response relationship for TF after affinity purification.

Figure 4 shows reversed phase hplc of affinity purified ferritin specific transfer factor.

Figure 5 shows reversed phase hplc of affinity purified ovalbumin specific transfer factor.

Figure 6 shows dose response relationships for reversed phase hplc purified transfer factor.

Figure 7 depicts analysis of reversed phase hplc fractions of ferritin specific transfer factor.

Figure 8 presents polytypic chromatography of affinity and reversed phase hplc purified transfer factor on gel filtration hplc columns.

Figure 9 presents activity data for **TF** for ferritin, from individual fractions of polytypic gel filtration hplc.

Figure 10 shows dose response relationships for the fraction described in Figure 8.

Figure 11 presents gel filtration chromatography of a **TF** fraction as in Figure 8.

Figure 12 shows a standard curve obtained from gel filtration chromatography of molecular weight markers.

Figure 13 shows the **UV** absorbance spectrum of a ferritin specific **TF**.

Figure 14 depicts antigen specificity for highly purified **TF**.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

This example explains the preparation of crude dialysates which contain transfer factor.

Following Petersen et al., J. Immunol 126: 2480-2484 (1981), groups of 100-150 **BALB/cByJ** mice, 8-14 weeks old, which had been maintained on water and pellet foot ad libitum, were "sensitized". This means that either ferritin or chicken egg albumin in aqueous solution was emulsified

in equal amounts of Hank's Balanced Salt Solution (HBSS), and Freund's complete adjuvant. Each mouse received 100 ug of the sensitizing antigen in a 40 ul volume, which was injected into two sites at the base of the tail, subcutaneously. After three weeks, six mice were selected randomly and were subjected to a delayed type hypersensitivity assay. This assay involved injection of 100 ug of antigen in 25 ul of HBSS, which was injected subcutaneously into hind footpads. Contralateral footpads were injected with 25 ul of HBSS. The antigen used in the assay was the same one administered to the mice previously. The footpad thickness was measured before and 18 hours after injection, using a dial gauge micrometer. Scores were taken from the difference between these values. Previous experimental work by Petersen et al., supra had shown that maximal swelling occurs 18-24 hours after injection.

If the subject mice had footpad swelling responses to the antigen significantly greater than the response to the diluent ($p<0.05$), all mice in the group were sacrificed. Spleens were removed aseptically, and single cell suspensions were prepared by gently forcing the cells through sterile 60 mesh stainless steel screens. The cells were washed three times with HBSS, an aliquot was removed, and mononuclear cells counted using trypan blue as vital exclusion dye. Overall viability was always greater than 90%. Cells were then suspended in sterile purified water in 50 ml sterile propylene centrifuge tubes, and lysed via repeated freezing in dry ice - ethanol baths and thawing in 37°C water baths. When microscopic observation confirmed that lysis was essentially complete, the lysates were placed in dialysis bags which had previously been boiled in purified water. These bags had molecular weight cut offs of 6000-8000. Dialysis was carried out at 4°C against 50 volumes of sterile purified water under constant stirring for 24 hours. This was conducted twice, serially. The dialysates which resulted were both pooled and lyophilized, with the lyophilized material reconstituted to 10^8

mononuclear cell equivalents (ce)/ml, using purified sterile water. After sterilization by passage through a 0.22 um filter, and confirmation of sterility with blood agar, the cells were stored at -20°C.

Example 2

The dialysates were assayed for TF activity as described by Kirkpatrick et al., J. Immunol 134: 1723-1727 (1985), the disclosure of which is incorporated by reference.

Samples were brought to 10^7 mononuclear ce/ml using sterile water as diluent. These materials were then administered to BALB/cByJ mice via intraperitoneal injection of 1.0 ml/mouse. These mice had not been "primed" by the antigen previously. Six mice were used for each data point. Delayed type hypersensitivity assays, as described supra, were conducted 24 hours later.

Results were quantitated by defining one unit of transfer factor activity as the material producing a half maximal footpad swelling response from a dose response curve of $\log_{10}$ of the mononuclear splenocyte equivalents versus footpad swelling increments. With the exception of crude dialysates, total protein levels in the preparations were so low that substantial proportions of the samples would be required for conventional protein assays. Thus, for this study, the number of units of transfer factor activity per absorbance unit at 214 nm is given.

This assay is the protocol used for all tests of activity described infra, whenever reference is made to an "in vivo transfer factor activity assay".

Example 3

This example describes affinity purification of transfer factor, following Kirkpatrick et al., J. Immunol. 135: 4027-4033 (1985), the disclosure of which is incorporated by reference. Immulon 2 Removawell strips were

filled with antigen at a 100-200 uM concentration in a 0.05 M sodium carbonate buffer, pH 9.6. Wells were incubated overnight at 4°C in a humid chamber, followed by washing three times with a **PBS-TWEEN** 20 solution (0.15 M **PBS**, pH 7.4, 0.5 ml **TWEEN**-20/liter). Bovine serum albumin was then added at a concentration of 100 mg/ml. The wells were incubated at room temperature for one hour to saturate remaining protein binding sites.

This was washed three more times with the **PBS-TWEEN** solution. Then, spleen cell dialysates containing transfer factor were applied at 10^8 mononuclear ce (cell equivalents)/ml and at a volume of 300 ul. Dialysates corresponded to the antigen that had been added previously - e.g., cell lysates from animals immunized with ferritin were used with ferritin treated strips. The strips were then incubated at 4°C, 24 hours in a humid chamber.

The wells were washed two more times with **PBS-TWEEN** 20, and then once more with **PBS**. Following this, 300 ul of acetonitrile were added, and the wells were incubated for ten minutes at room temperature. Supernatants were removed, and an amount corresponding to 2.4×10^8 ce (2.4 ml) were said aside for the in vivo transfer factor assay described supra. Samples were dried under nitrogen in a 37°C water bath. The samples for the assay were reconstituted to 10^7 ce/ml, using purified water. The material used in further purification steps was dissolved in from 1-5 ml of 5 mM ammonium bicarbonate. The use of this material is described infra.

Example 4

Affinity purified **TF** was then applied to reversed phase hplc. Between 10 and 30 x 10^8 ce were dissolved in a 0.2 to 0.5 ml volume of purified water, and this was applied to a 4.6 x 250 mm Vydac, 218TP54 octadecylsilane column, using 5.0 mM ammonium bicarbonate at flow rate 1.0 ml/min. Fractions were collected at 1 minute intervals, and UV data permitted detection. This was done via **UV** spectral data

taken over 1.0 second intervals, between 203 and 280 nm, and monitoring absorbance at 214 nm.

Results not elaborated upon here showed that when 5 mM ammonium bicarbonate and acetonitrile were used, with acetonitrile ranging from 0 - 60% of the eluate, all **TF** activity eluted in the void volume. As a result, elution was carried out isocratically using 5 mM ammonium bicarbonate, and the unretained peak collected. Aliquots, usually containing 2.5 x 10^8 ces were set aside for **TF** in vivo transfer factor assays. The remainder was lyophilized, reconstituted using 1.0 ml of 10 mM formic acid, and retained at -20°C for further purification by polytypic hplc on gel filtrate columns.

Example 5

This example is directed to the purification of transfer factor using polytypic hplc on gel filtration columns.

To accomplish this, 20-30x10^8 ces of mononuclear cells were applied in 0.2 to 0.5 ml volumes to two 7.8 x 300 mm gel filtration hplc columns, linked in series. This was eluted with 10 mM formic acid, pumped at a flow rate of 0.5 ml/mm. This system had a void volume of 12.2 ml,and fractions were collected at 1.0 minute intervals, detection being accomplished as indicated supra.

Example 6

Comparative data were obtained by using the ion pairing agent **TBAP** and a 4.0x300 mm octadecylsilane column for reversed phase hplc. Gradient elution in pilot experiments using 5 mM **TBAP** as starting solvent and 80% acetonitrile as final solvent indicated that all **TF** eluted prior to a 25% (v/v) acetonitrile concentration. Linear gradients were performed using 5 mM **TBAP**/acetonitrile (92:8; v/v) as solvent **"A"** and 5 mM **TBAP**/acetonitrile (75:25; v/v) as solvent **"B"**. Gradients were of the form: 0% **B** (10 minutes),

0-100% B (5 minutes) and 100% B (5.5 minutes). Flow rate was 0.5 ml/min, detection being carried out as described supra.

Example 7

A molecular weight determination was carried out using an adaptation of gel filtration hplc methodology developed by Meyerson et al, Peptides 7: 481-489 (1986). Individual samples were passed through two 7.8x300 mm gel filtration hplc columns linked in series, using 50 mM potassium phosphate buffer, pH 7.0 with 200 mM NaCl as eluant. Samples were dissolved either in eluant or 1.0 mM HCl, depending on solubility. Empirically determined flow rate of 0.49 ml/min was used for eluant. The void volume was 12.0 ml (24.4 min), and total permeation volume of 22.5 ml (46.0 min).

Example 8

A microdialysis method was used to analyze purified **TF** preparations. It was a modification of Overall, Anal. Biochem. 165: 208-214 (1987). Spectra/por 7 dialysis tubing was cut into small squares and washed in purified water. Potential peptide binding sites were saturated by incubating rinsed dialysis membranes at 4°C for 16 hours with 0.1% (w/v) Na_3N solution containing 25 ug/ml of a glutamic acid terpolymer of Mr 405, together with 25 ug/ml of peptide **LWMRFA**, Mr 823. Supernatant was discarded, and purified water added to the tube, followed by moderate shaking. Rinsing was repeated at least 8 times.

Following this, a cap of a 1.5 ml microcentrifuge tube was punctured using the wide end of a heated Pasteur pipet. Samples for dialysis ranging from 200 to 1000 ul were placed in the tubes, and dialysis membrane pieces placed across the open end. The cap was closed, and the tube inverted and fixed, using tape to the inner wall of a dialysis chamber containing 500 ml pure water. Trapped air was removed using

"u" or hook shaped tipped Pasteur pipets, covered with a small piece of tubing.

Dialysis was carried out at 4°C under constant stirring for from 2-6 hours, depending on sample volume. Dialysate was discarded and, as needed, the above was repeated. Microcentrifuge tube was removed and centrifuged for 10 seconds in a microcentrifuge. Sample was carefully removed using a sterile tipped micropipette.

Example 9

Dose-response studies were carried out using the splenocyte dialysates and affinity purified materials described supra. In these experiments, the foot pad delayed-type hypersensititivty assay described supra was carried out. Groups of 6 mice were used for each data point, and the testing was performed by injecting the antigen 24 hours after i.p. injection of the sample. Responses were taken 18 hours after this.

Background footpad response represents mice which received no i.p. sample. This is represented by "0 ce" in Figures 2 and 3. Coefficients of determination are expressed by r^2.

Figures 2 and 3 present these data. In each case, "A" represents results obtained using ovalbumin specific transfer factor, and "B" ferritin specific transfer factor.

In these data, magnitude of footpad swelling was proportional to the $\log_{10}$ of the dose when crude dialysates were used. This was previously observed by Rozzo et al., Cell Immunol. 115: 130-145 (1988). Coefficients of determination (r^2) were 0.97 (Figure 2A) and 0.99 (Figures 2B); therefore, the data do describe the relationship well.

As shown in Figure 3, the curves were similar, but the coefficients of determination were lower, being 0.80 for Figure 3A, and 0.82 for 3B.

Example 10

Following the purification protocols described supra, yield and specific activity were calculated, also as indicated supra. These results are presented in Table 1, which follows. Ovalbumin transfer factor showed a 46-fold enhancement of specific activity with a 66% yield, while ferritin transfer factor gave values of 53 fold and 59%.

In Table 1, "RPLC" refers to reversed phase liquid chromatography, and "GFC" to the polytypic hplc on gel filtration columns.

Table I

Yield and Specific Activities for Purified Materials

Experimental details can be found under "Materials and Methods". A unit of transfer factor activity is defined as the amount of transfer factor-containing sample (expressed in the number of mononuclear cell equivalents (ce) from which it was derived) required to produce a one-half maximal footpad swelling response. Specific activity is defined as the number of units of transfer factor activity per absorbance unit at 214nm.

PREPARATION	UNITS $_{TF}$ per 10^7 ce	AU $_{214nm}$ per 10^7 ce	UNITS $_{TF}$ per AU_{214nm}	TOTAL CELL EQUIVALENTS ce (10^{-8})	TOTAL UNITS T.F. ACTIVITY ce (10^{-8})	INDIVIDUAL YIELD (%)	CUMULATIVE YIELD (%)
Ovalbumin Transfer Factor (Pool 73)							
Dialysate	1,370	2.770	495	484	66.3	–	–
Affinity Purified	910	0.040	22,800	470	42.8	66	66
Fraction A (RPLC)	910	0.037	24,600	465	42.3	100	66
Fraction AIII (GFC)	770	0.035	22,000	463	35.7	85	56
Ferritin Transfer Factor (Pool 76)							
Dialysate	1,020	2.340	436	145	14.8	–	–
Affinity Purified	600	0.026	23,100	143	8.58	59	59
Fraction A (RPLC)	1,270	0.020	63,500	130	16.5	212	125
Fraction AIII (GFC)	600	0.018	33,300	123	7.38	47	50

Example 11

Affinity purified material, as described supra, was subjected to chromatographic analysis using rplc incorporating 5 mM **TBAP** as ion pairing agent. **"TBAP"** refers to a tetrabutylammonium phosphate based solvent system. In these experiments, 43.2×10^8 ces were applied in a 400 ul volume. With reference to example 6, supra, fractions were analyzed. The major chromophore detected at 214 nm. (Fraction 3; time=26.4 minutes) contained **TF** activity. Fractions 1 and 5 also showed the activity at much lower levels, as can be seen via reference to Figure 4, showing data obtained using ferritin specific **TF**. The activity was measured using the previously mentioned footpad assay.

These results caused focus to be placed on fraction 3.

Example 12

The elution profiles of affinity **TFs** were obtained, a typical one being shown in Figure 5. To obtain this, 23.3×10^8 ce of affinity purified albumin specific **TFs**, using rplc were applied in 50 ul volumes. All transfer factor activity eluted in unretained peak (fraction A), while contaminants were retained. **TF** activity was measured using the above mentioned footpad assay. Fraction A showed $17.33 \pm 1.20 \times 10^{-2}$ mm ($p<0.001$) swelling, whereas fraction B (impurities) showed $5.5 \pm 1.61 \times 10^{-2}$ mm, with p being not significant.

Example 13

The fraction A described supra was assayed and showed only a 7% enrichment in specific activity. Yield, however was 100%, as shown in Table I. These data were confirmed by studies which showed that rechromatography of lyophilized, reconstituted fraction A sample showed essentially the same unretained peak.

Example 14

Fraction A type materials were obtained for ferritin specific **TF**, just as ovalbumin specific **TF** was obtained. This fraction showed 2.75 fold enrichment (Table I); however, there was an apparent two fold (212%) yield over affinity purified sample, and 125% yield relative to crude dialysate. The reason for these data is not known.

Example 15

Dose response curves for the "fraction A" referred to supra for both ovalbumin and ferritin specific **TFs** were obtained, as set out in Example 9, supra. The results are depicted in Figures 6A and 6B (ovalbumin and ferritin, respectively). Coefficients of determination are 0.96 and 0.97, respectively.

Example 16

Fraction A ferritin specific **TF** material was analyzed using a **TBAP** system. 10.5×10^8 ce were applied to the column in a 100 ul volume. The analysis, as indicated by Figure 7, contained four components which eluted at 4.7, 16.1, 21.3 and 26.4 minutes. **TF** activity was found in the last of these. After correcting for solvent baseline absorbance characteristics of the gradient, this corresponds to approximately 90% of the 214 nm absorbing material.

Example 17

Fraction A material was purified further, using polytypic gel filtration hplc. To do so, 25.4×10^8 ce were applied to the columns in volumes of 200 ul. The eluant was 10 mM formic acid, and an elution profile, for ferritin specific **TF** fraction A is shown in Figure 8. Fraction "AIII", i.e., the 3rd fraction to elute, contained all of the **TF** activity and was studied further.

Example 18

Ferritin specific TF fraction AIII from the gel filtration hplc was analyzed, by neutralizing 50 ul aliquots from individual fractions with 50 mM ammonium bicarbonate, and diluted with sterile, purified water to $1.8x10^6$ ce/ml. Activity was analyzed for each fraction. "Pre" fractions represent a pool of fractions 24 through 32, and "post' fractions 47-60. TF activity was found only in fractions 39-42 (Figure 9).

Example 19

Dose response curves were derived for "Fraction AIII" materials, (both types), as done in prior experiments. These results are shown in Figures 10A and 10B for ovalbumin and ferritin specific TF, respectively. For ovalbumin, the coefficient of determination r^2 was 0.86, and one unit of activity per $1.3x10^4$ ce. Ferritin specific TF showed r^2 of 0.99, and one unit per $1.68x10^4$ ce. These results lead to the conclusion that spleens from mice given a single sensitizing dose of antigen and containing $0.5-2.0x10^8$ mononuclear leukocytes would yield $3.8x10^3$ to $1.5x10^4$ units of TF for ovalbumin. The data for ferritin would suggest $3x10^3$ to $1.5x10^4$ units for comparable mice, wherein the purification scheme of Figure 1 is used.

Specific activity of the ovalbumin specific fraction was 11% less than the fraction A material, but the yield was 85%, suggesting 44 fold enrichment. With respect to the ferritin specific fraction, specific activity was 48% lower than fraction A, but 1.4 fold higher than affinity purified material, and 76 fold higher than the dialysate. Yield was 47%, giving a cumulative yield of 50%.

Example 20

Gel filtration hplc of Meyerson et al., supra was used to analyze the purity of fraction AIII and to determine molecular weight. The methodology of gel filtration is

described supra, and will not be repeated here. Figure 11 depicts the results. TF activity for ferritin specific material coincided with elution volume of 17.98 ml (fraction b). Footpad responses by recipients of $4x10^7$ ce for fraction a were $5.83\pm2.31x10^{-2}$ mm (p insignificant) and $19.50\pm1.82x10^{-2}$ mm ($p<0.001$) for fraction b. The peak representing fraction b contains 98% of the 214 nm absorbing material.

Given that low recovery is frequently observed when small quantities of protein are dialyzed, materials from the peaks were desalted, via modification of Overall, supra. The material was then tested for transfer factor activity, giving the results previously mentioned in this experiment.

To determine molecular weight, the same system was used to obtain the standard curve shown in Figure 12, for which data were obtained by using molecular weight markers in separate runs. The analysis yields an estimated molecular weight of Transfer Factor of about 4900 to about 5500.

Example 21

An ultraviolet spectrum taken for fraction b, is depicted in Figure 13. These data were taken from $2.5x10^8$ ce, and show relatively little absorbance at wavelengths greater than 235 nm. This includes wavelengths classically used in monitoring transfer factor, including 254 nm, 260 nm, and 280 nm.

This means that the transfer factors isolated herein are peptides or proteins, without nucleic acid components. Chromatography solvents which permit the analysis at lower wavelengths allow for this.

Example 22

Antigen specificity of the purified AIII fraction TFs was studied. Mice were injected with a TF preparation produced in response to one of either ovalbumin or ferritin, (10^6 ce in 1.0 ml), followed by challenge 24 hours later

with ovalbumin and ferritin. Neither preparation induced response to the heterologous antigen, but both showed the delayed hypersensitivity reaction with the homologous material, indicating that the transfer factor retained antigen specificity (Figure 14).

The transfer factor-containing dialysates described herein showed very similar specific activity (ovalbumin: 495 units at 214 nm; ferritin: 436 units at 214 nm), indicating extremely potent preparations. The data of Table 1 suggest that the spleen of one sensitized mouse, containing about 10^8 mononuclear leukocytes produces enough transfer factor to transfer significant delayed type hypersensitivity to at least 10^3, and perhaps as many as 10^4 nm unsensitized recipients.

The affinity purification step, i.e., where **TF** is reacted with its antigen, causes a loss of about 40% of transfer factor activity, but enhances specified activity by about 50-fold. Thus the purified **TF** is extremely specific.

Affinity purified **TF**, when used in an NH_4HCO_3 based system, was eluted in void volume eluate, indicating the highly polar nature of the material. Data obtained for affinity purified ovalbumin specific transfer factor shows a slight increase in specific activity, and no loss of active material. The ferritin specific **TF** gave less predictable results. The 2.75 fold increase in specific activity, taken with an apparent yield of 213%, coupled with decrease in **UV** absorbency, may suggest, inter alia, that an inhibitor of the transfer factor was removed. Indeed, Rozzo et al., Cell Immunol. 115: 130-145 (1988); Borkowsky et al., in Kirkpatrick et al., ed., Immunobiology of Transfer Factor pg. 91-115 (Academic Press, 1983), and Gottlieb, U.S. Patent Nos. 4,616,079 and 4,468,379, suggest existence of such factors.

It has been noted, supra that earlier work postulated an oligonucleotide residue as part of the transfer factor

molecule. While it is possible that the described process removed this residue, it would not account for the absence of significant 280 nm absorbance, nor would it account for the retention of biological activity. Thus, it appears that antigen specific transfer factors are peptide molecules having a molecular weight of from about 4.9 to about 5.5 kilodaltons. These transfer factors are produced in sensitized animals in extremely small, but extremely potent amounts.

The process for producing the purified transfer factor involves a number of steps. First, the sample containing the transfer factor is filtered such as by dialysis, to separate materials having low molecular weight, i.e., less than about 15 kilodaltons, from other materials. This low molecular weight fraction contains the transfer factor. This fraction is then affinity purified by contacting it to antigen against which the transfer factor is specific, so as to complex the transfer factor from the fraction. Following separation of the transfer factor from the antigen, it is applied to a first reversed phase, high performance liquid chromatography column and is eluted therefrom. The eluate from the first column is applied to gel a filtration high performance liquid chromatography column, and is eluted with a second eluant. This eluate contains the proteinaceous transfer factor.

The first eluant is preferably ammonium bicarbonate, but need not be, as long as the eluant is a substance in which the transfer factor is soluble. The second eluant is preferably a volatile solvent such as a volatile acid. Formic acid is particularly preferred. The sample analyzed, of course contains the transfer factor molecule. Examples of samples that contain transfer factor are materials which contain cells of lymphoid origin, such as splenocytes, peripheral blood leukocytes, lymph node cells, and thoracic duct cells. If necessary or desirable, samples can be treated to lyse these cells prior to separation.

In separating the transfer factor from the sample, the antigen may be, e.g., bound to a solid phase matrix, covalently or non-covalently.

The purified transfer factor thus obtained is characterized as a peptide of from about 4900-5500 daltons, which transfers delayed type hypersensitivity or other expressions of cell mediated immune processes to a specific antigen to a non-sensitized individual, when the transfer factor is administered to a non-sensitized individual. The transfer factor does not affect antibody mediated responses, nor does it induce antibody production.

As has been shown, the prior art molecules consisting of peptide and oligonucleotide have been implicated in treatment of many pathological conditions. It has been shown herein that the purified proteinaceous transfer factor does transfer the delayed type hypersensitivity to a specific antigen. Thus, the invention embraces the treatment of pathological conditions where an immune response is needed or an immune deficiency must be corrected via administering an amount of the transfer factor to a non-sensitized individual in an amount sufficient to provoke expression of cell mediated immunity against an antigen.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

We claim:

1. Process for obtaining purified antigen specific transfer factor comprising:

(i) contacting a transfer factor containing sample to a filtering means having a molecular weight cut off point of less than about 15 kilodaltons to obtain a fraction containing proteins having a molecular weight of less than about 15 kilodaltons;

(ii) contacting said fraction to an antigen against which said transfer factor is specific under conditions favoring complexing of antigen specific transfer factor to said antigen;

(iii) separating said antigen specific transfer factor from said antigen;

(iv) applying said antigen specific transfer factor to a first reversed phase, high performance liquid chromatography column;

(v) applying a first eluant to said first reversed phase, high performance liquid chromatography column to remove said antigen specific transfer therefrom, in the form of an eluate;

(vi) applying said elute to a second, gel filtration, high performance liquid chromatography column, and

(vii) eluting purified, antigen specific transfer factor from said second, gel filtration high performance liquid chromatography column with a second eluant.

2. Process of claim 1, wherein said first eluant comprises a solution of ammonium bicarbonate.

3. Process of claim 1, wherein said second solvent is a volatile solvent.

4. Process of claim 3, wherein said volatile solvent is a solution of a volatile acid.

5. Process of claim 4, wherein said volatile acid is formic acid.

6. Process of claim 1, wherein said sample contains lymphoid cells.

7. Process of claim 6, wherein said lymphoid cells comprise splenocytes, peripheral blood leukocytes, lymph node cells, or thoracic duct cell.

8. Process of claim 1, wherein said antigen is covalently or non-covalently bound to a solid phase matrix.

9. Process of claim 2, wherein said ammonium bicarbonate solution has a concentration of 5 mM.

10. Process of claim 5, wherein said solution of formic acid has a concentration of about 10 mM.

11. Substantially pure antigen specific transfer factor prepared by the process of claim 1.

12. Substantially pure antigen specific transfer factor characterized as a protein having a molecular weight of from about 4900 to about 5500 daltons, and further characterized by (i) affecting transfer of delayed type cell mediated immune response to an antigen, (ii) not affected antibody mediated antigen response, and (iii) not affecting induction of antibody production.

13. Method for treating a pathological condition comprising administering to a subject with a pathological condition an amount of the substantially pure, antigen specific transfer factor of claim 9 sufficient to induce an immune response or to correct an immune deficiency, said transfer factor having been obtained from a second subject

who expresses cell mediated immunity against said pathological condition.

FIG. 1.

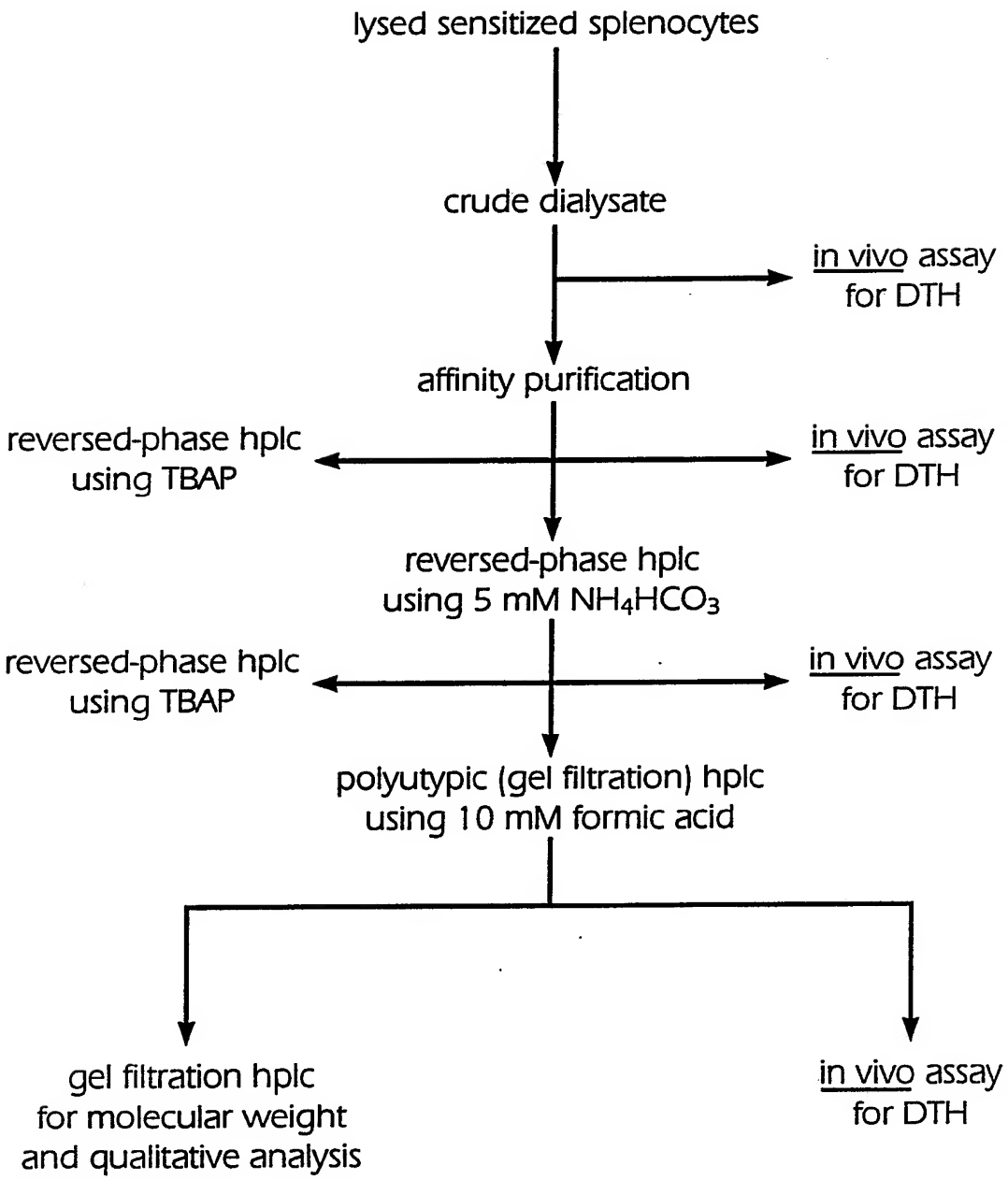

lysed sensitized splenocytes
crude dialysate
in vivo assay for DTH
affinity purification
reversed-phase hplc using TBAP
in vivo assay for DTH
reversed-phase hplc using 5 mM NH_4HCO_3
reversed-phase hplc using TBAP
in vivo assay for DTH
polyutypic (gel filtration) hplc using 10 mM formic acid
gel filtration hplc for molecular weight and qualitative analysis
in vivo assay for DTH

FIG. 2A.

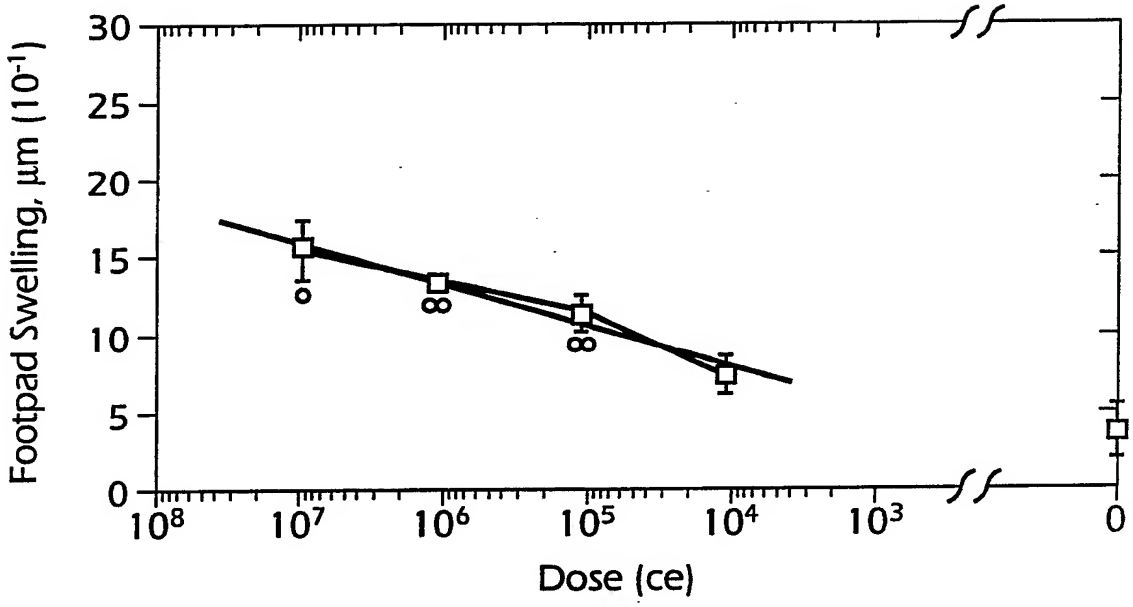

Footpad Swelling, μm (10^{-1})
30
25
20
15
10
5
0
10^8
10^7
10^6
10^5
10^4
10^3
0
Dose (ce)

FIG. 2B.

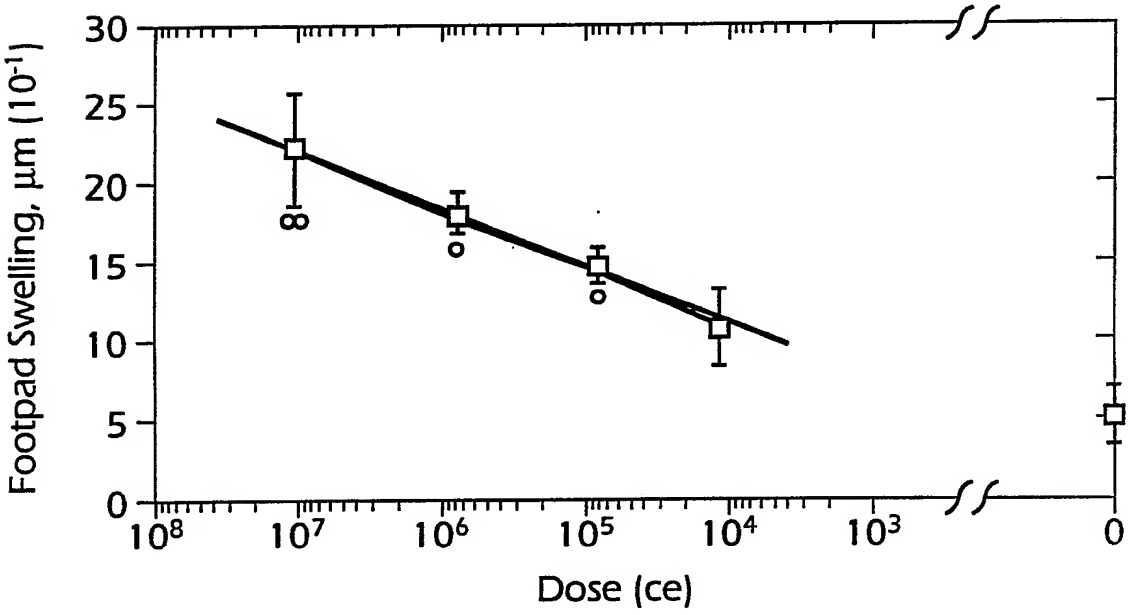

Footpad Swelling, μm (10^{-1})
30
25
20
15
10
5
0
10^8
10^7
10^6
10^5
10^4
10^3
0
Dose (ce)

FIG. 3A.

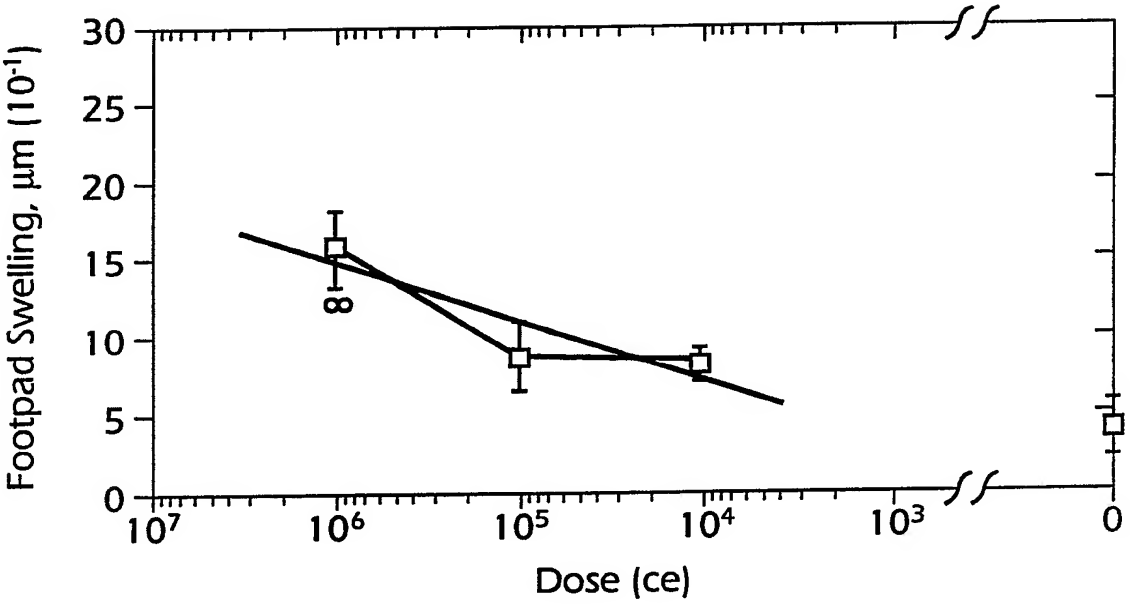

Footpad Swelling, μm (10^{-1})
30
25
20
15
10
5
0
10^7
10^6
10^5
10^4
10^3
0
Dose (ce)

FIG. 3B.

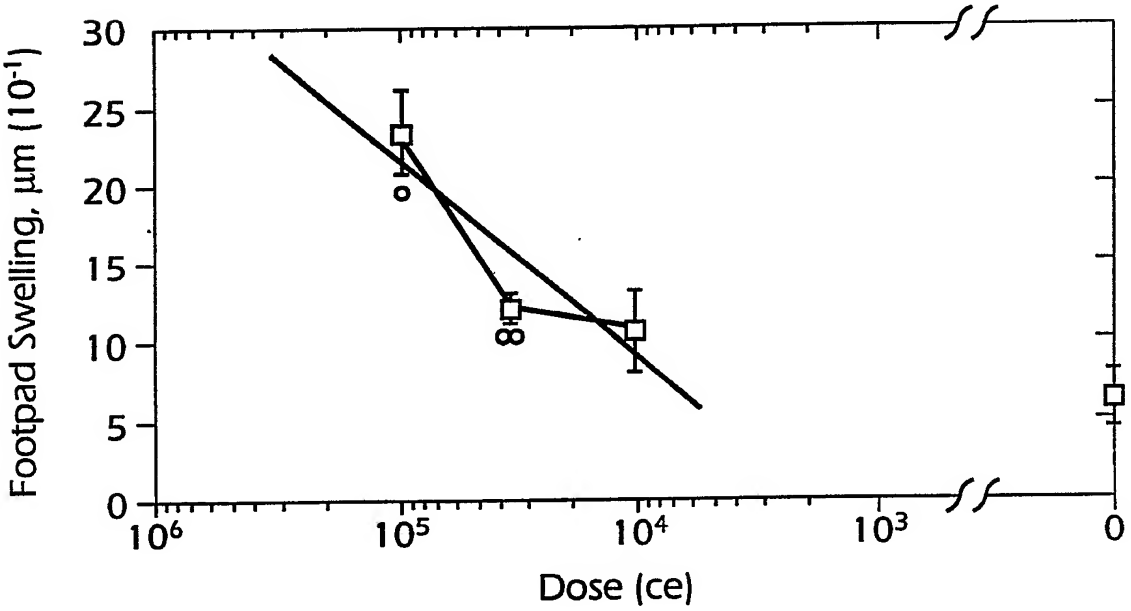

Footpad Swelling, μm (10^{-1})
30
25
20
15
10
5
0
10^6
10^5
10^4
10^3
0
Dose (ce)

FIG. 4.

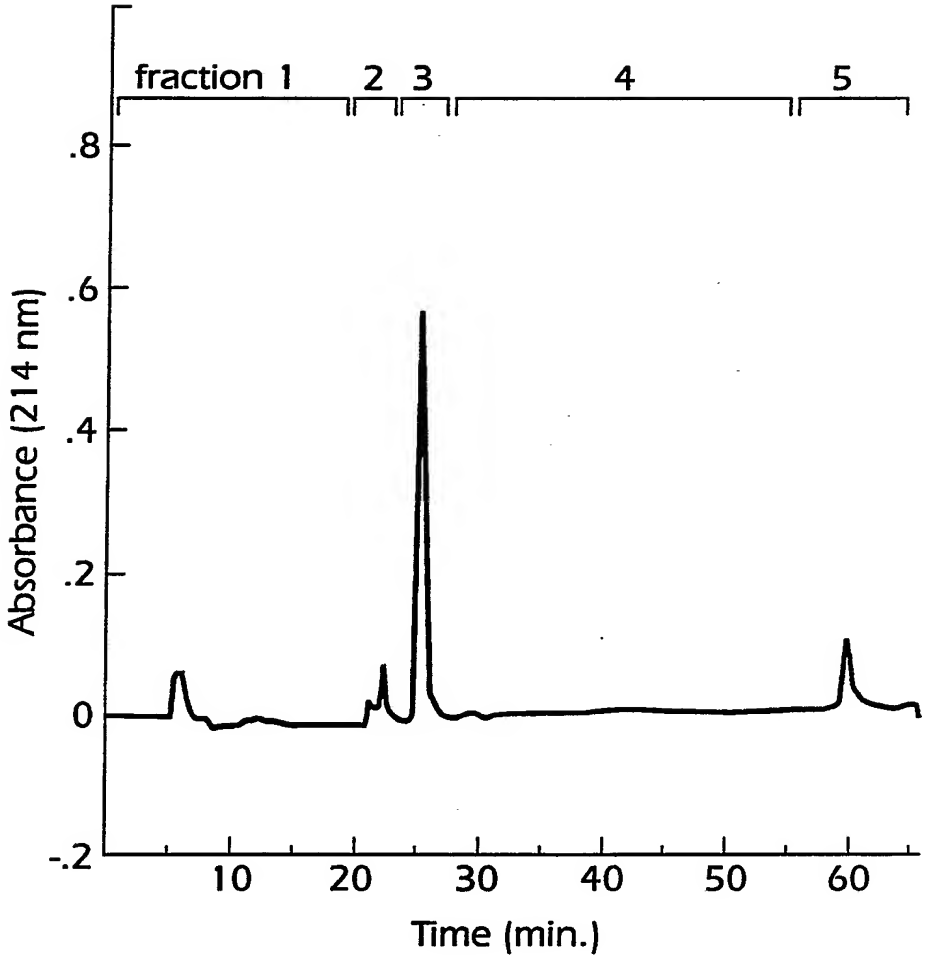

fraction 1
2
3
4
5
.8
.6
.4
.2
0
-.2
Absorbance (214 nm)
10
20
30
40
50
60
Time (min.)

FIG. 5.

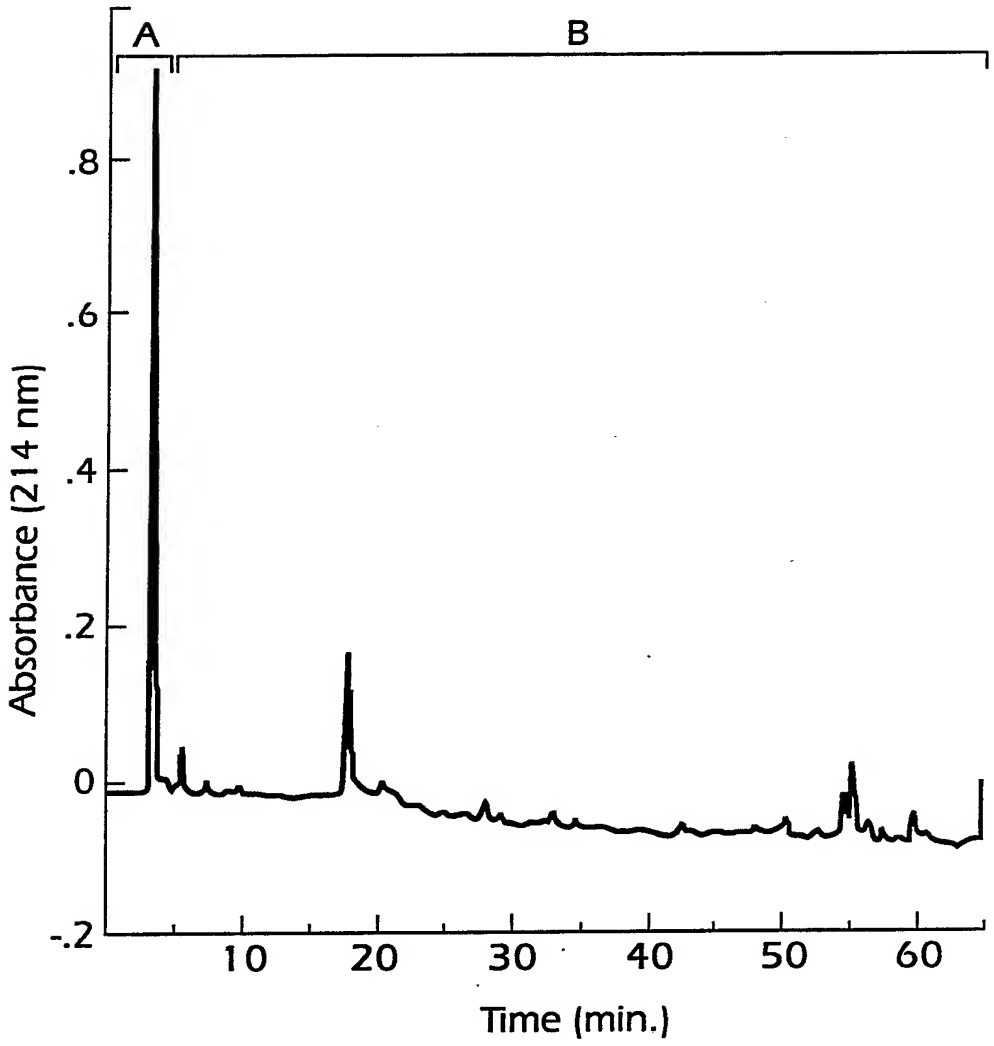


FIG. 6A.

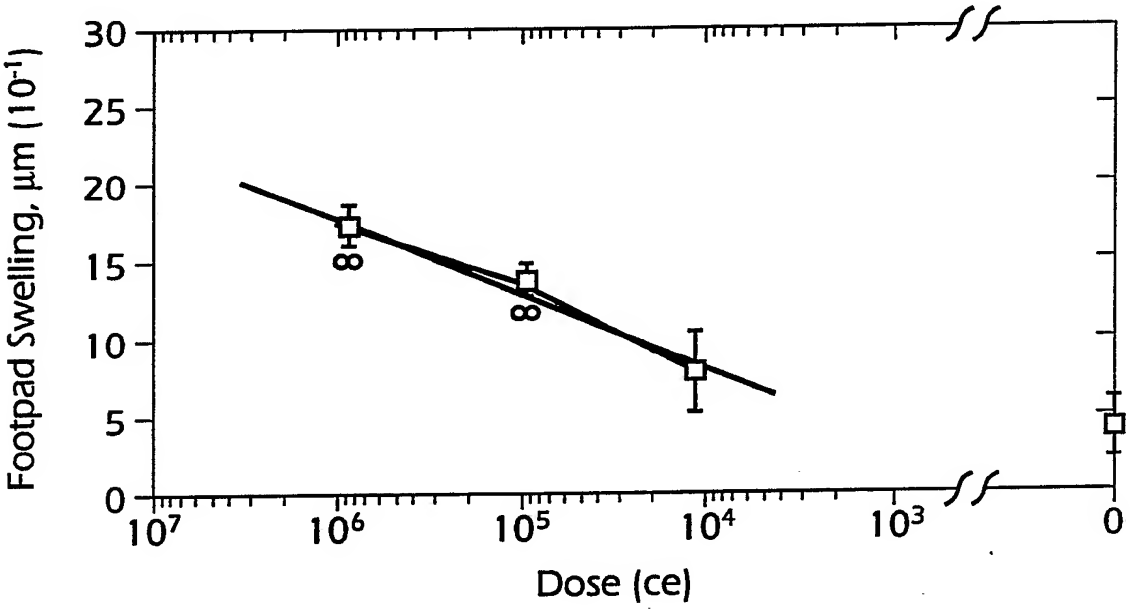

Footpad Swelling, μm (10^{-1})
30
25
20
15
10
5
0
10^7
10^6
10^5
10^4
10^3
0
Dose (ce)

FIG. 6B.

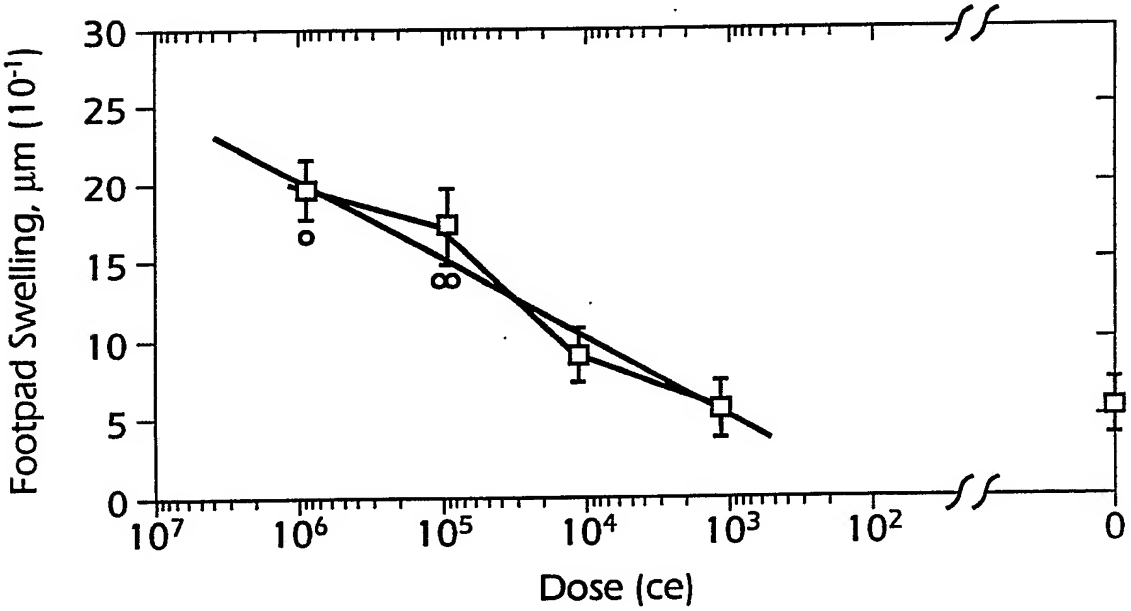

Footpad Swelling, μm (10^{-1})
30
25
20
15
10
5
0
10^7
10^6
10^5
10^4
10^3
10^2
0
Dose (ce)

FIG. 7.

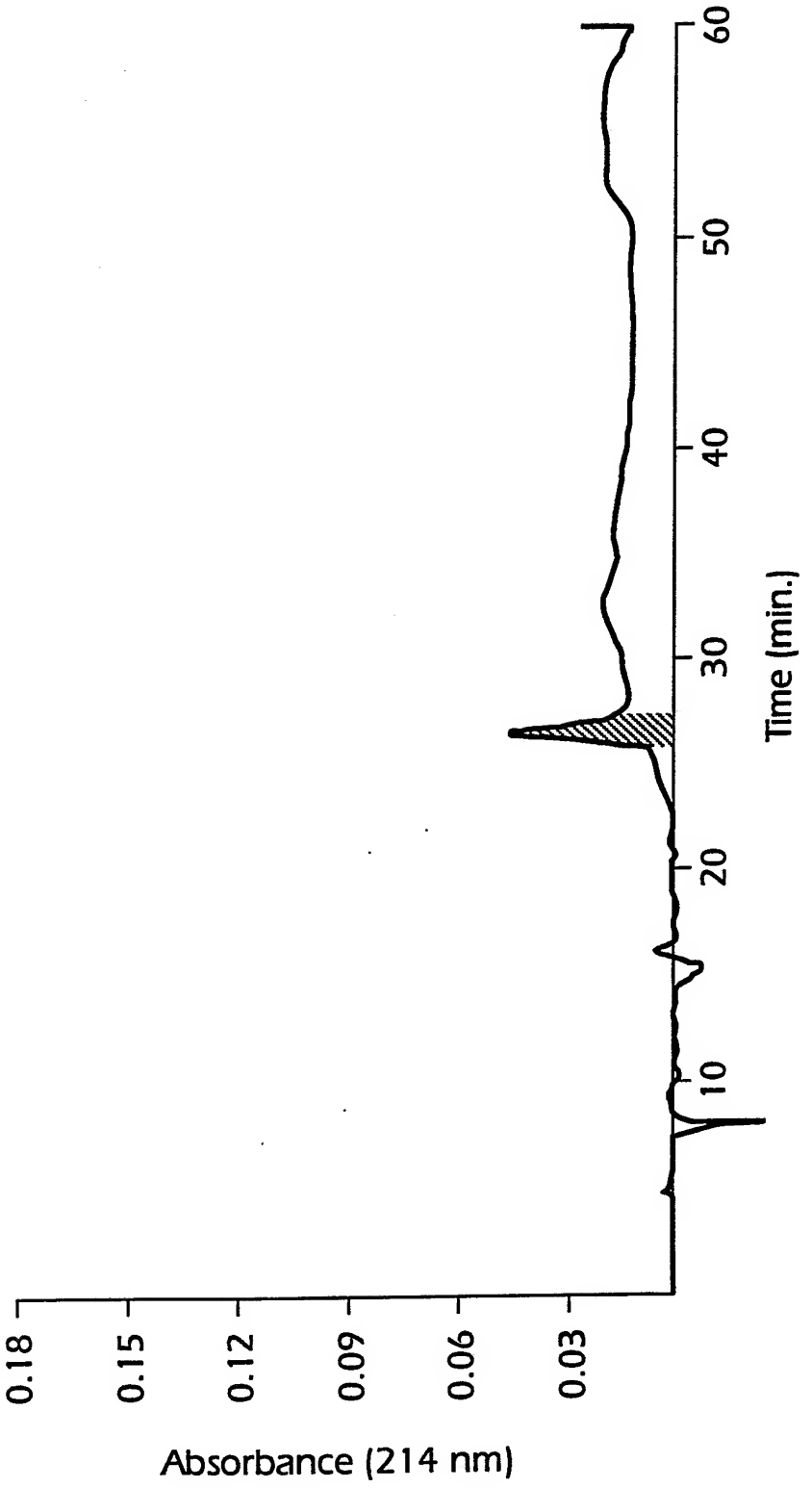

0.18
0.15
0.12
0.09
0.06
0.03
Absorbance (214 nm)
10
20
30
40
50
60
Time (min.)

FIG. 8.

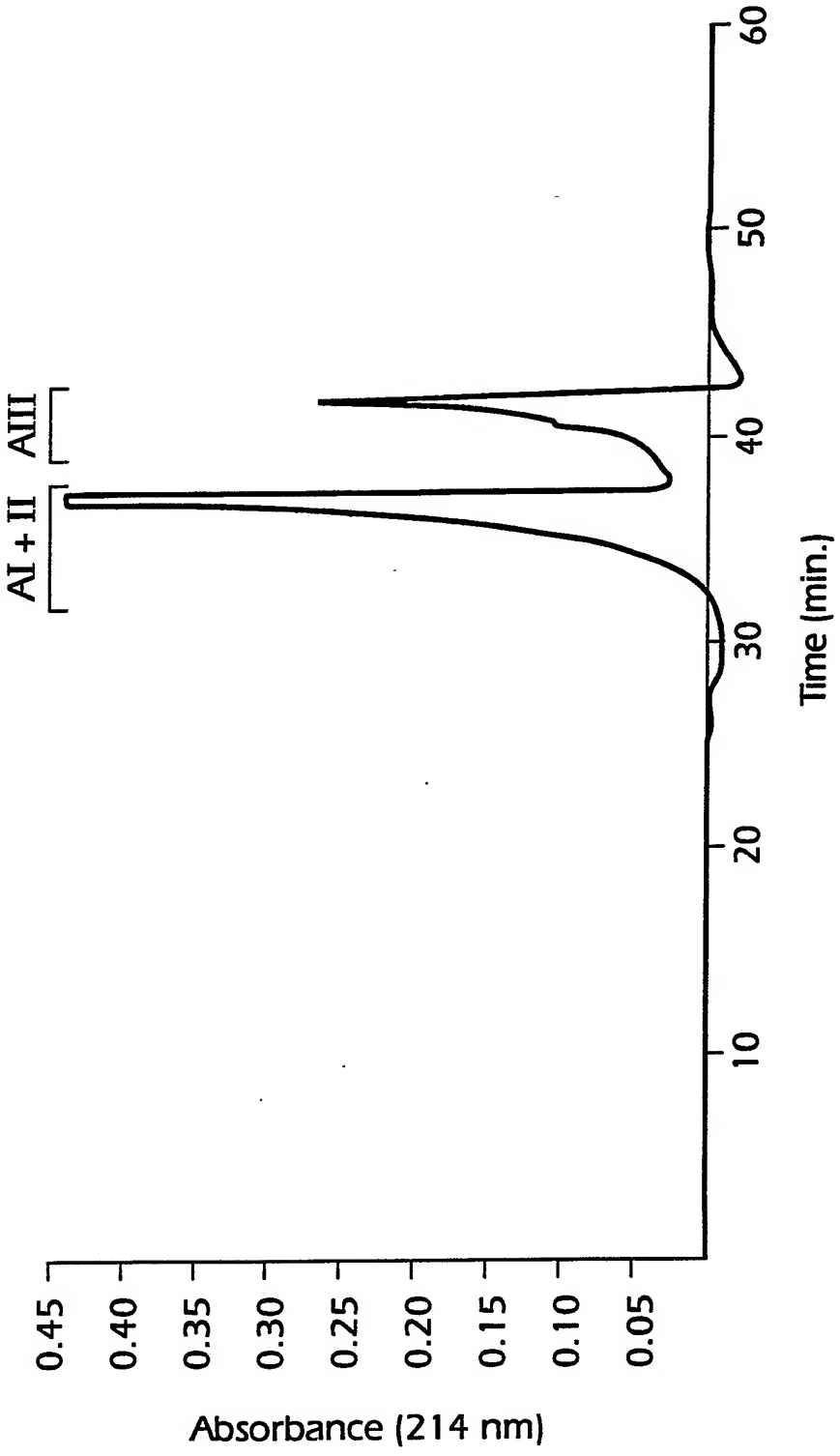

AI + II
AIII
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
Absorbance (214 nm)
10
20
30
40
50
60
Time (min.)

FIG. 9.

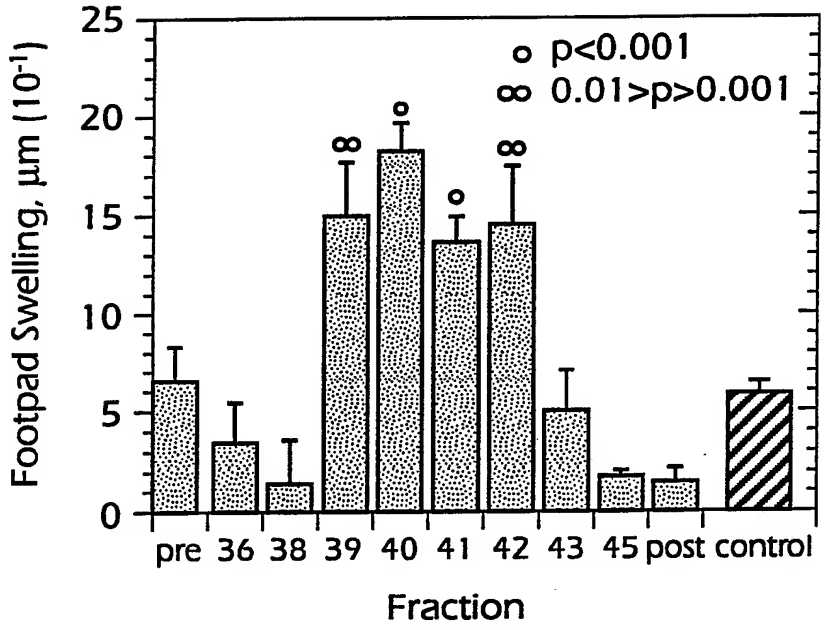

Footpad Swelling, μm (10^{-1})
25
20
15
10
5
0
o p<0.001
∞ 0.01>p>0.001
pre 36 38 39 40 41 42 43 45 post control
Fraction

FIG. 12.

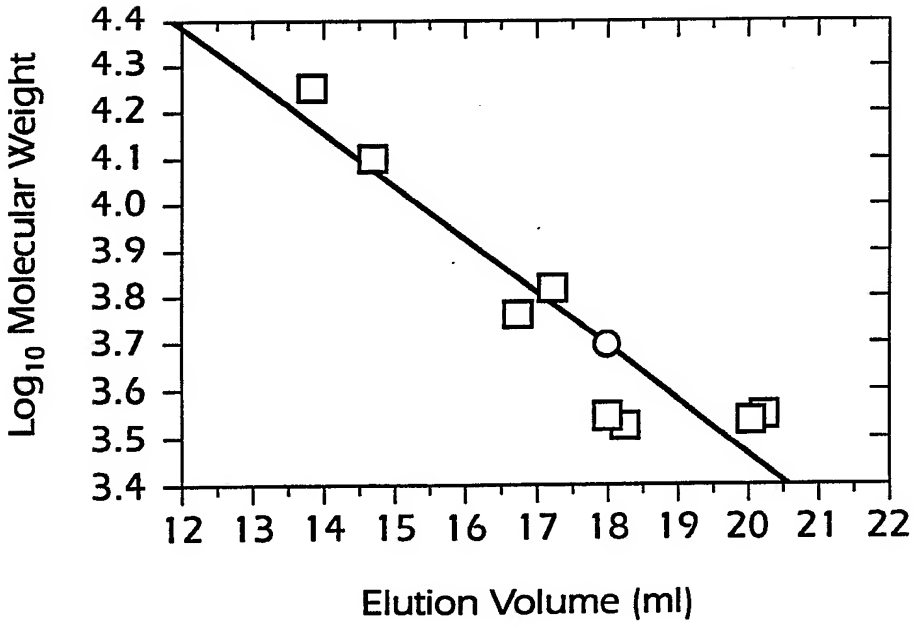

Log_{10} Molecular Weight
4.4
4.3
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
12 13 14 15 16 17 18 19 20 21 22
Elution Volume (ml)

FIG. 10A.

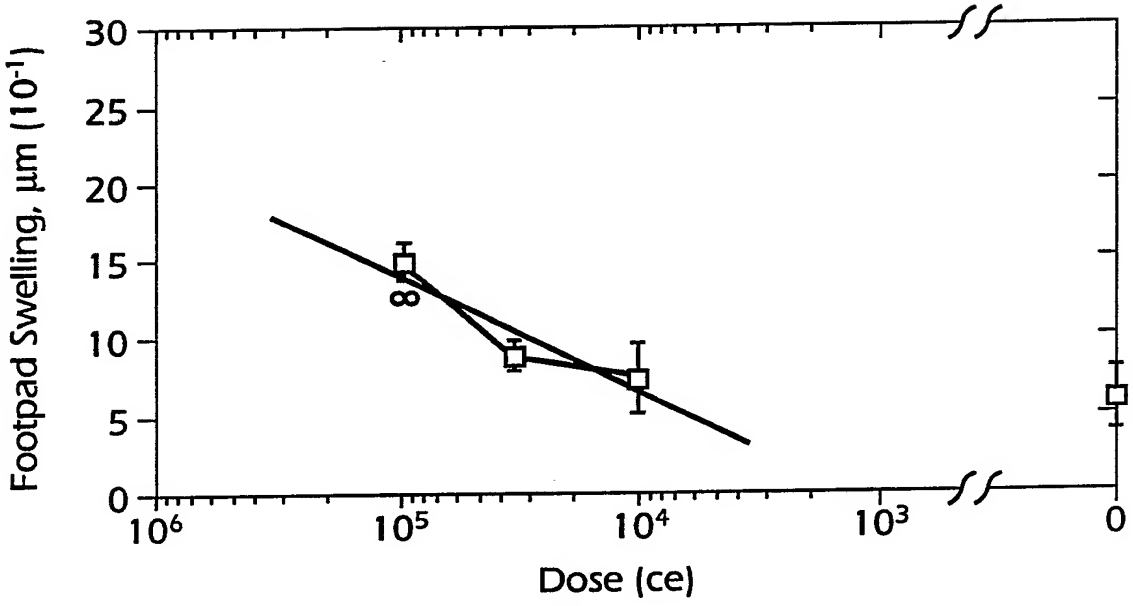


FIG. 10B.

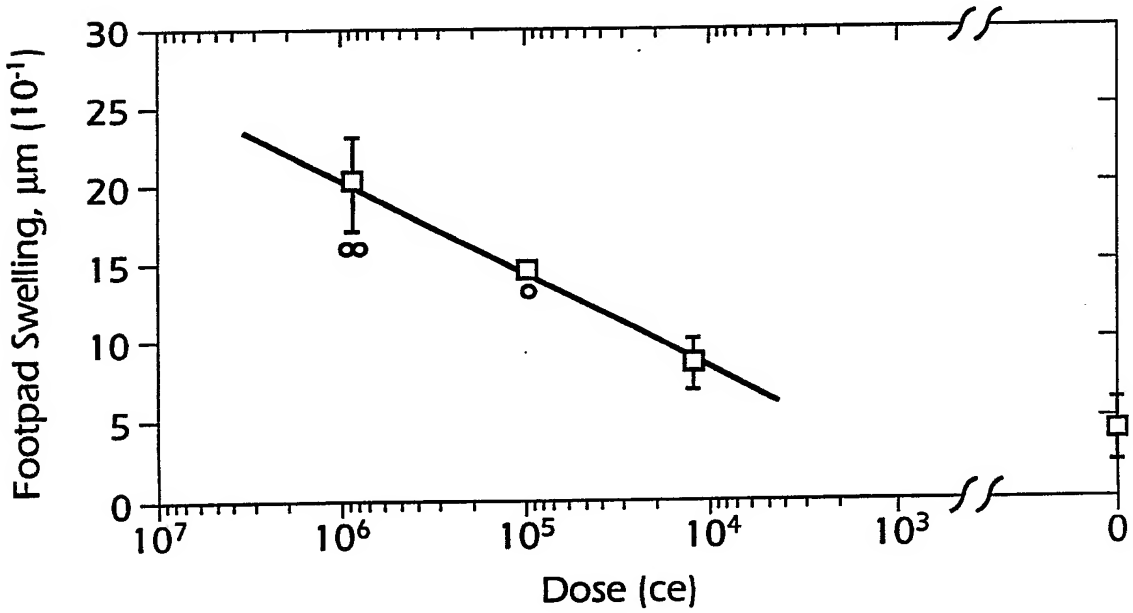


FIG. 11.

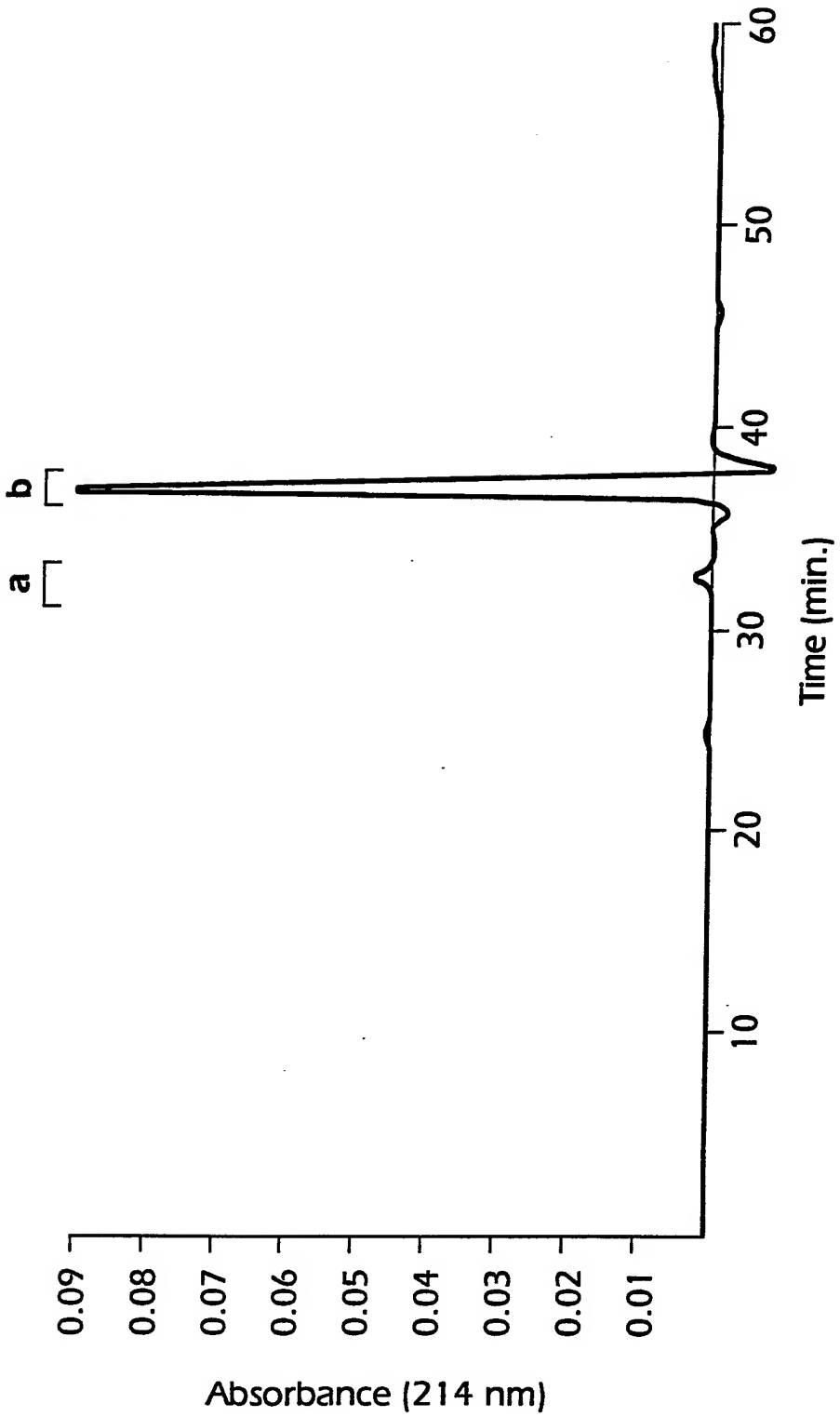

b
a
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Absorbance (214 nm)
10
20
30
40
50
60
Time (min.)

FIG. 13.

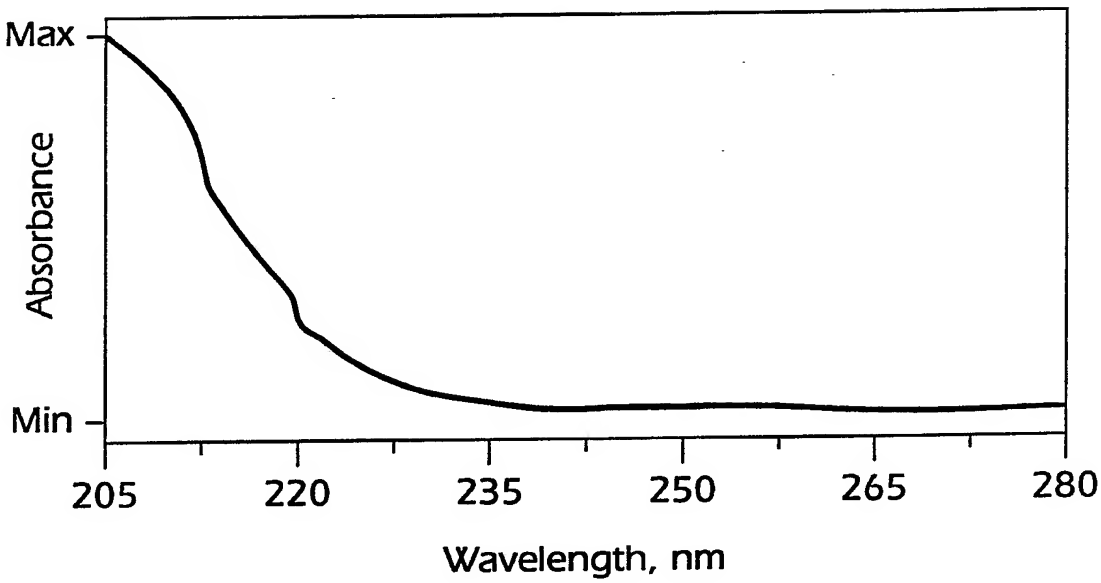
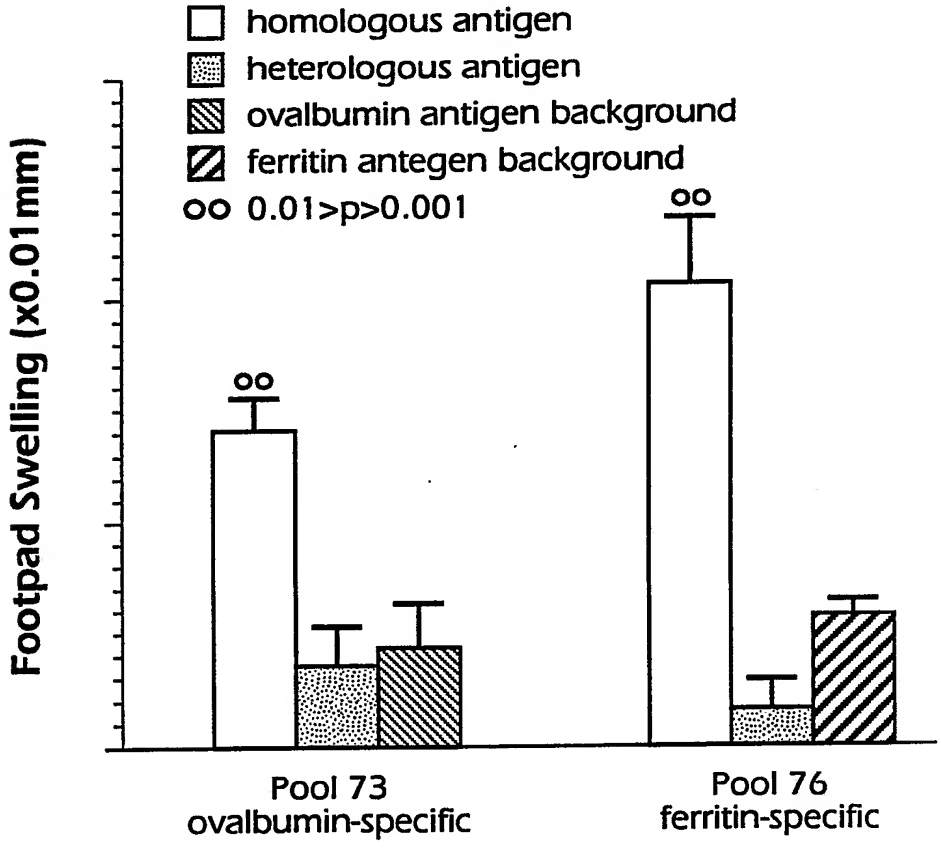

Max
Absorbance
Min
205
220
235
250
265
280
Wavelength, nm

FIG. 14.


homologous antigen
heterologous antigen
ovalbumin antigen background
ferritin antegen background
oo 0.01>p>0.001
Footpad Swelling (x0.01mm)
oo
oo
Pool 73
ovalbumin-specific
Pool 76
ferritin-specific
Transfer Factor Preparation

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04118

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/00; C07K 3/00
U.S.Cl.: 530/344, 380, 417; 514/21

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.:	530/344,380,417; 514/21

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y Y	US,A, 4,468,379 (GOTTLIEB) 28 August 1984, see columns 3, 8, 9.	1-12 12
Y Y	The Journal of Immunology, Volume 135, No. 6, issued December 1985, C.H. Kirpatrick et al., "Murine Transfer Factor III. Specific Interactions Between Transfer Factor and Antigen", pages 4027-4033, see entire article.	1-12 12
A	US,A, 3,991,182 (SPITLER ET AL.) 09 November 1976, see entire patent.	1-12

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 August 1991	20 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Bennett Celsa

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP,A, 0,101,200 (YEDA ET AL.) 22 February 1984, see entire patent.	1-13
A	US,A, 4,616,079 (GOTTLIEB) 07 October 1986, see entire patent.	1-13
A	The Journal of Immunology, Volume 134, No. 3, Issued March 1985, C.H. Kirpatrick et al., "Murine Transfer Factor, II. Transfer of Delayed Hypersensitivity to Synthetic Antigens", pages 1723-1727, see entire document.	1-13